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Photoreceptors Regulating Circadian Behavior: A Mouse Model

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Abstract: Our recent studies have examined circadian photoreception in mice with hereditary retinal disorders (*rd/rd* and *rds/rds*). Despite the loss of visual function in these mice, circadian responses to light remain unaffected. Using c-fos expression within the SCN as a marker of neural activation of the circadian entrainment pathway, we find identical levels of Fos in the SCN of *rd/rd* and +/+ mice in response to retinal illumination. On the basis of action spectrum studies, and measurements of photopigment retinoids using HPLC, we believe the photopigment mediating circadian responses to light is based upon an opsin, and that 11-cis-retinaldehyde is the photopigment chromophore. Preliminary measurements of mouse rod opsin, blue cone, and green/red cone opsin mRNA in retinally degenerate mice suggest that none of these opsins are exclusively used to mediate circadian responses to light. Collectively our data suggest that circadian photoreception can be maintained by a very small number of rod or cone cells without outer segments, or alternatively, is performed by an unrecognized class of photoreceptive cell within the mammalian retina.

For a circadian oscillator to be of any use to an organism, internal time must be synchronized to environmental time, and for the majority of circadian rhythms the biological clock is primarily entrained by

photoreceptors that detect the irradiance changes associated with dawn and dusk. Although it is clear that photoreceptors play a crucial role in the organization of circadian physiology, our understanding of these sensory cells remains surprisingly superficial. In many cases these photoreceptive cells, and the nature of their connections with the oscillator, remain unknown. In contrast to the rest of the vertebrates, which have both pineal and deep brain photoreceptors (Foster, Garcia-Fernandez, Provencio & DeGrip, 1993), mammals use their eyes for all forms of photoreception (Foster, Timmers, Schalken & De Grip, 1989). However, it remains unclear which cells within the eye mediate circadian responses to light.

Our recent studies have employed mice with specific genetic lesions as "reduced preparations" with which to address questions about photic regulation of the circadian system in mammals. These studies have shown that the involvement, if any, of retinal rods and/or cones in mediating circadian responses to light is complex (Foster, et al., 1991). Mice homozygous for the autosomal recessive allele *rd* (retinally degenerate) experience a massive degeneration of the visual cells. This defect has been associated with an accumulation of cGMP in the *rd/rd* mouse rod photoreceptors due to a mutation in the gene encoding the ß-subunit of the rod-specific phosphodiesterase (Bowes, et al., 1990). By 60 days of age all rod cells have degenerated, and between 90 and 150 days of age even the crudest electrophysiological and behavioral visual responses to bright light have been reported to disappear (Carter-Dawson, LaVail & Sidman, 1978). Approximately 98% of mouse photoreceptors are rods, and although all the rods degenerate in the *rd/rd* retina, a few cone cells remain in mutants over one year of age.

The sensitivity of the photoreceptors mediating phase shifts was

determined by measuring the magnitude of phase shift in freerunning locomotor activity in response to exposure of 15 minutes of monochromatic light (515 nm) of various irradiances. These experiments were conducted using three genotypes (*rd/rd, rd/+, +/+*) of the same strain (C57/BL) 80-100 days of age. Despite the loss of visual photoreceptors in *rd/rd* mice, these animals show circadian responses to light that are indistinguishable from mice with phenotypically normal retinas (*rd/+, +/+*). It is important to stress that not only does some photosensitivity remain in *rd/rd* mice, but the circadian photosensitivity shown by these animals is not different from the sensitivity of animals with intact retinas. In addition, the site of circadian photoreception must reside within the eye because bilateral enucleation of *rd/rd* animals abolishes all circadian responses to light (Foster, et al., 1991).

The progression of rod degeneration in *rd/rd* mice commences early in post-natal development, followed by a more protracted loss of cone cell bodies. If the surviving cone cells mediate circadian responses to light, then one might expect the sensitivity of the circadian system to light in *rd/rd* mice to decline with age. Circadian responses to light of *rd/rd* and *+/+* mice up to 800 days of age have been examined. The results indicate that the phase shifts of *rd/rd* and *+/+* mice are indistinguishable, and therefore, circadian responses to light do not parallel cone cell loss (Provencio, Tennant, Card & Foster, 1992).

Previous studies suggest that *rd/rd* mice become blind between 90 - 150 days of age. However the behavioral assays used in these experiments tested for fairly complex visual tasks (Carter-Dawson, et al., 1978). We have started our own series of experiments to assess the "visual" capabilities of mice with retinal degeneration. A transparent chamber

separated by a black partition with a small window was constructed. A mild electric shock could be administered to the mouse in either half of the chamber. The mouse could avoid the shock by jumping into the other half of the chamber. The first experiments determined how quickly *rd/rd* and +/+ mice would learn to escape the shock. Both genotypes learned to avoid the shock after only a few trials, establishing that the *rd/rd* and +/+ mice had identical learning abilities. The next series of experiments tested whether *rd/rd* mice would anticipate an electric shock if the shock was preceded with a light signal. Mice, 80-100 days of age, were placed in the chamber in the dark. The animal was illuminated for 10 seconds (white light at 10 $\mu W.cm^{-2}$) before receiving the shock. To avoid the shock the animal must perceive the light, associate this with the shock, and jump into the other half of the chamber. The results in **Figure 1** show that while +/+ mice could perceive the light and anticipate the shock, *rd/rd* mice could not. These preliminary data indicate that even the crudest visual light detection task seems to be absent in *rd/rd* mice. These data would be consistent with the hypothesis that visual photoreceptors are not required for circadian regulation. This view has been strengthened by our recent studies on Fos induction within the murine suprachiasmatic nuclei (SCN).

Several laboratories have shown that the activation of the immediate early gene *c-fos* within the SCN is a useful marker of neural activation of the light input pathway of the circadian system (Kornhauser, Nelson, Mayo & Takahashi, 1990). If the rods and cones of the retina contribute to light information reaching the SCN, one might expect a lower expression of c-*fos* in the SCN of *rd/rd* versus +/+ mice after retinal illumination. If, however, circadian photoreception is not affected by this

mutation, then light activated c-*fos* expression should be the same in the SCN of both genotypes. By localizing the protein product of the c-*fos* gene, Fos, we have been unable to detect any differences in the distribution, number and density of labeled SCN cells between the two genotypes after retinal illumination (Colwell & Foster, 1992).

The literature reports that there are no outer segments in the *rd/rd* retina (Carter-Dawson, et al., 1978). As a result we concluded that circadian photoreception is not being mediated by a cell with an outer segment. To support this hypothesis, the circadian responses of mice homozygous for the retinally degenerate slow (*rds*) mutation have been examined. Mice carrying this mutation never form rod or cone outer segments, and photoreceptor cell bodies slowly die. These animals lack a specific glycoprotein (peripherin) important for outer segment disc assembly (Travis, Sutcliffe & Bok, 1991). If outer segments are required for full circadian photoreception, then the light sensitivity of the circadian system of *rds/rds* animals would be attenuated by this mutation. Using the same assay of circadian light detection used to examine the photosensitivity of the *rd* mutant, the effects of a light pulse in phase shifting the circadian system were examined in +/+, *rd/rd* and *rds/rds* mice of the C3Hf strain, ranging in age from 7 - 14 months. Circadian responses were identical in all three genotypes (**Figure 2**).

Collectively, these data suggest that circadian photoreception can be maintained by a small number of cone cells without outer segments, or alternatively performed by an unrecognized class of photoreceptive cell that is unaffected by the *rd/rd* mutation, and that functions normally to regulate rhythmic physiology and behavior. Currently a range of techniques are being used to distinguish between these alternatives, addressing two

overlapping questions: 1) What is the circadian photopigment, and 2) Where is the circadian photoreceptor localized within the retina? The approach taken has been to identify the photopigment, and then localize this photopigment within the retina.

An action spectrum for phase-shifting the circadian rhythm in *rd/rd* and +/+ mice, aged between 80-100 days, is in progress and the preliminary data suggest that the wavelength of maximum sensitivity is near 500 nm. In addition, the shape of the action spectrum suggests a typical animal photopigment based upon an opsin and 11-*cis*-retinaldehyde chromophore. HPLC techniques have identified significant amounts of 11-*cis*-retinaldehyde within the dark adapted eyes of *rd/rd* mice (1-2 % of the levels found in +/+ individuals). If this chromophore is associated with the residual rods and cones of the degenerating *rd/rd* retina, then one would expect to see a decline in chromophore that would parallel the loss of the rods and cones, and levels should ultimately disappear. Alternatively, if the chromophore is associated with circadian responses to light, which do not decline with age, then one might expect chromophore to remain at a constant low level. The results show that after an initial rise and decline during the first 14 days after birth, chromophore levels remain constant in the *rd/rd* eye and parallel circadian responses to light.

Dr. Meredith Applebury's laboratory (University of Chicago) has successfully characterized the genes and cDNAs for the rod opsin, blue cone opsin, and green/red cone opsin in the +/+ mouse. Because circadian responses to light and photopigment chromophore remain stable in aged *rd/rd* mice we would anticipate that any opsin mediating circadian responses to light would also remain at a constant low level. Preliminary

Northern blot analysis using Applebury's probes has shown that rod opsin mRNA rapidly disappears from the eye, followed by the slower decline of blue cone opsin and green/red cone opsin mRNA levels. If these preliminary results are correct, and all the known mouse opsins do decline with age, then this leaves us with essentially two alternatives: 1) None of the known mouse opsins mediate circadian responses to light, and there is a unique "circadian" opsin within the eye, or 2) one or more of these opsins mediates circadian responses to light and occurs at very low levels within the retina. We aim to use the more sensitive RNase protection assays to help resolve these alternatives.

The light detecting system used to entrain rodent circadian physiology seems to be based upon an insensitive photon counting mechanism which integrates light inputs over relatively long duration's (Nelson & Takahashi, 1991). We have shown that the loss of rods and cones in mice with *rd* and *rds* retinal mutations parallels a decline in visual function, yet circadian responses to light remain unaffected in these animals. Interpretation of these data is complex, but is consistent with the hypothesis that intact retinal rods and cones do not normally play a significant role in the regulation of circadian physiology by light, and that some other photoreceptor within the eye performs this task. While we are inclined to favor this novel interpretation, a range of alternatives exist.

While rod and cone photoreceptor loss is reported to be compete by 12 months of age in *rds* mice (Sanyal, De Ruiter & Hawkins, 1980), we cannot totally exclude the possibility that a few surviving photoreceptor cell bodies survive in aged mice and that these cells are "sufficient" to maintain circadian responses to light. If circadian photosensitivity were directly related to photoreceptor number, then one would expect to

observe a decline in circadian responses to light as photoreceptors are lost from the retina (**Figure 3a**). If, however, the output from circadian photoreceptive elements is averaged, then a progressive loss of circadian sensitivity would not be observed (**Figure 3b**). Such a system would require relatively few photoreceptive cells to show normal responses to light. In this case the sensitivity-limiting step is "down stream" from the photoreceptors. If the "averaging processor" (**Figure 3b**) requires a high number of counts from the photon counters, requiring many photons for a minimal response, then a few photoreceptive elements would be sufficient to maintain sensitivity near threshold light levels. This requirement for high light levels (many photons entering the eye) will ensure that even very few photoreceptors will encounter sufficient quanta to elicit a response. If threshold levels were very low, requiring few photons for a minimal response, then a few photoreceptors would not be sufficient to maintain sensitivity near threshold. In this case the low light levels around threshold (few photons entering the eye), would not ensure a sufficient number of quantal hits. As has been noted previously, the threshold for circadian responses to light is typically high relative to the threshold for visual responses (Nelson & Takahashi, 1991). So it is possible that the circadian light detecting system employs some form of averaging processor with a high photic threshold. Such a system would be buffered from the degenerative loss of photoreceptive elements in the *rd* and *rds* eye. Only when photoreceptor loss was almost complete would a decline in sensitivity be noted.

One could also speculate that a decline in photoreceptor number and loss of outer segments could be in some way compensated by either synaptic reorganization or some form of up-regulation of transduction

processes. For example, one way to increase sensitivity would be to inactivate the inhibitory protein arrestin. Arrestin blocks the ability of light-activated opsin to stimulate the G protein transducin. Whatever the mechanism for up-regulation, the amount of up-regulation would have to be substantial, compensating for both the loss of almost all photoreceptive cells and complete loss of outer segments. The implementation of such large scale compensatory mechanisms is not impossible but seems to us unlikely.

The identification of the photoreceptors which mediate circadian responses to light in mammals remains a mystery. While this photoreceptor system is clearly different from the visual system, we cannot yet say whether there is some unidentified circadian photoreceptor within the eye, or whether a few rod and or cone cells, with no outer segments, are sufficient to perform this task. Our future studies are directed towards resolving these various alternatives.

Figure 1: Experiments to assess the "visual capabilities" of *rd/rd* and +/+ mice. This figure shows one example of an age matched pair of mice (*rd/rd* and +/+, 80 days of age). Animals were placed in a chamber in the dark and then illuminated for 10 seconds (white light at 10 $\mu W.cm^{-2}$) before receiving a mild electric shock. To avoid the shock the animal must perceive the light, associate this with the shock, and make an avoidance response. Animals were tested 20 times each day over a period of approximately one hour. The number of times the mouse anticipated the shock was recorded. The results show that while +/+ mice (black bars) could perceive the light and learn to anticipate the shock, *rd/rd* mice (open bars) could not. These preliminary data indicate that even the crudest visual tasks seem to be absent in *rd/rd* mice which show normal circadian responses to light.

Figure 2: Comparison of the effect of a 60-70% saturating, 15 min. light pulse (515 nm; 1.3 x 10^{-1} $\mu W.cm^{-2}$) in phase-shifting the freerunning circadian rhythm of C3Hf mice with normal retinas (+/+) and degenerate retinas (*rd/rd* & *rds/rds*). Animals range in age from 6 - 14 months. These data show that circadian phase-shifts in response to a standard light pulse have been unaffected by either form of retinal degeneration.

Figure 3: Graphical models for possible photic information flow through the rodent circadian system. (**a**) Model showing a direct relationship between photoreceptor number and sensitivity. In this model the "processor" adds all photic input. As a result, the loss of photoreceptors would be directly proportional to the sensitivity of the system. (**b**) Model in which a direct relationship between photoreceptor number and sensitivity does not exist. Because information from the photon counters is averaged by the processor, loss of photon counters (photoreceptors) would not directly lead to a loss in the sensitivity of the system. Only when photoreceptor number decreased to a level when quantal hits failed to occur would a drop in sensitivity be noted. It is possible that the circadian light detecting system employs some form of averaging processor with a high photic threshold. Such a system would be buffered from the degenerative loss of photoreceptive elements in the *rd* and *rds* retinal mutants (see text for details).

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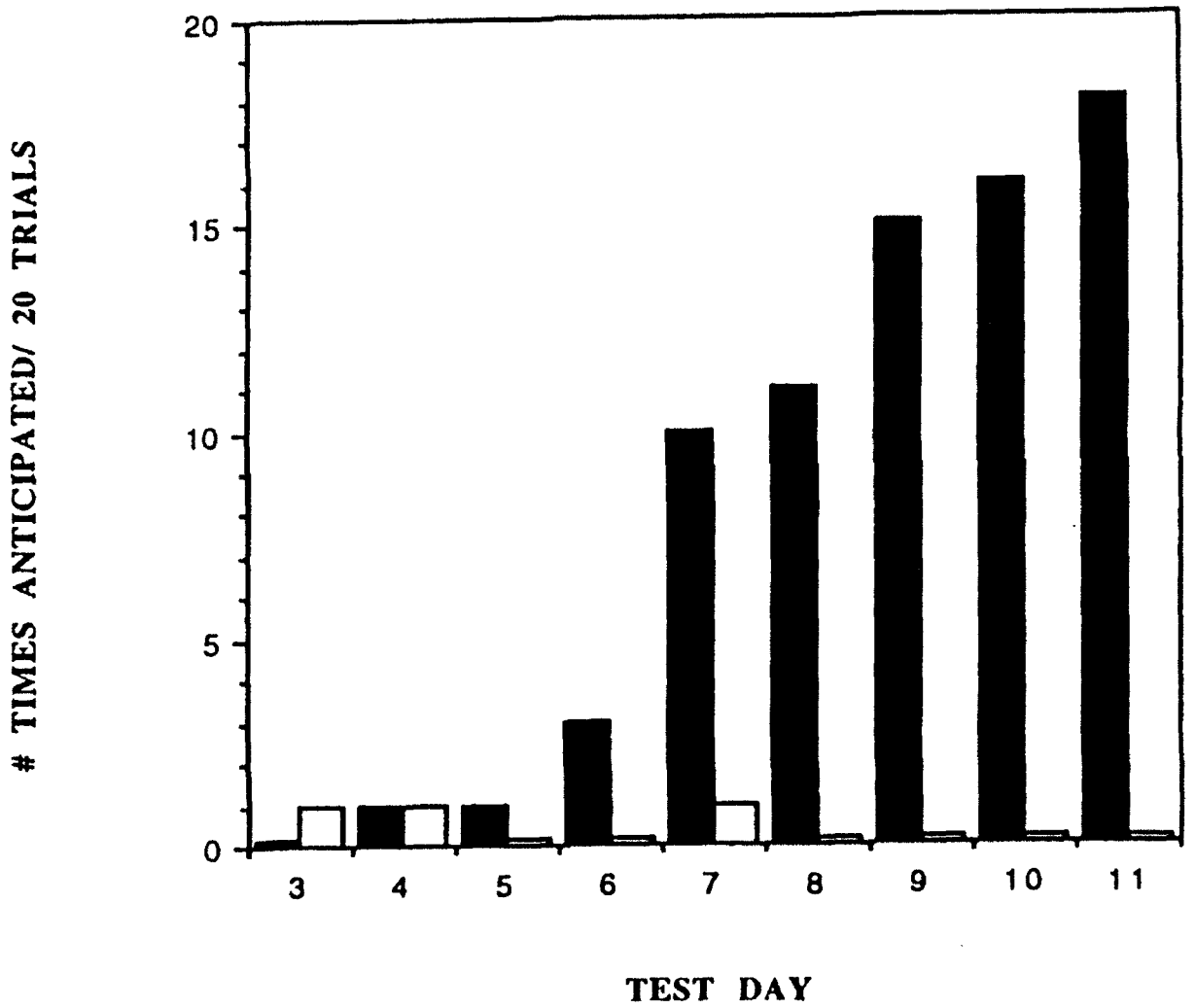
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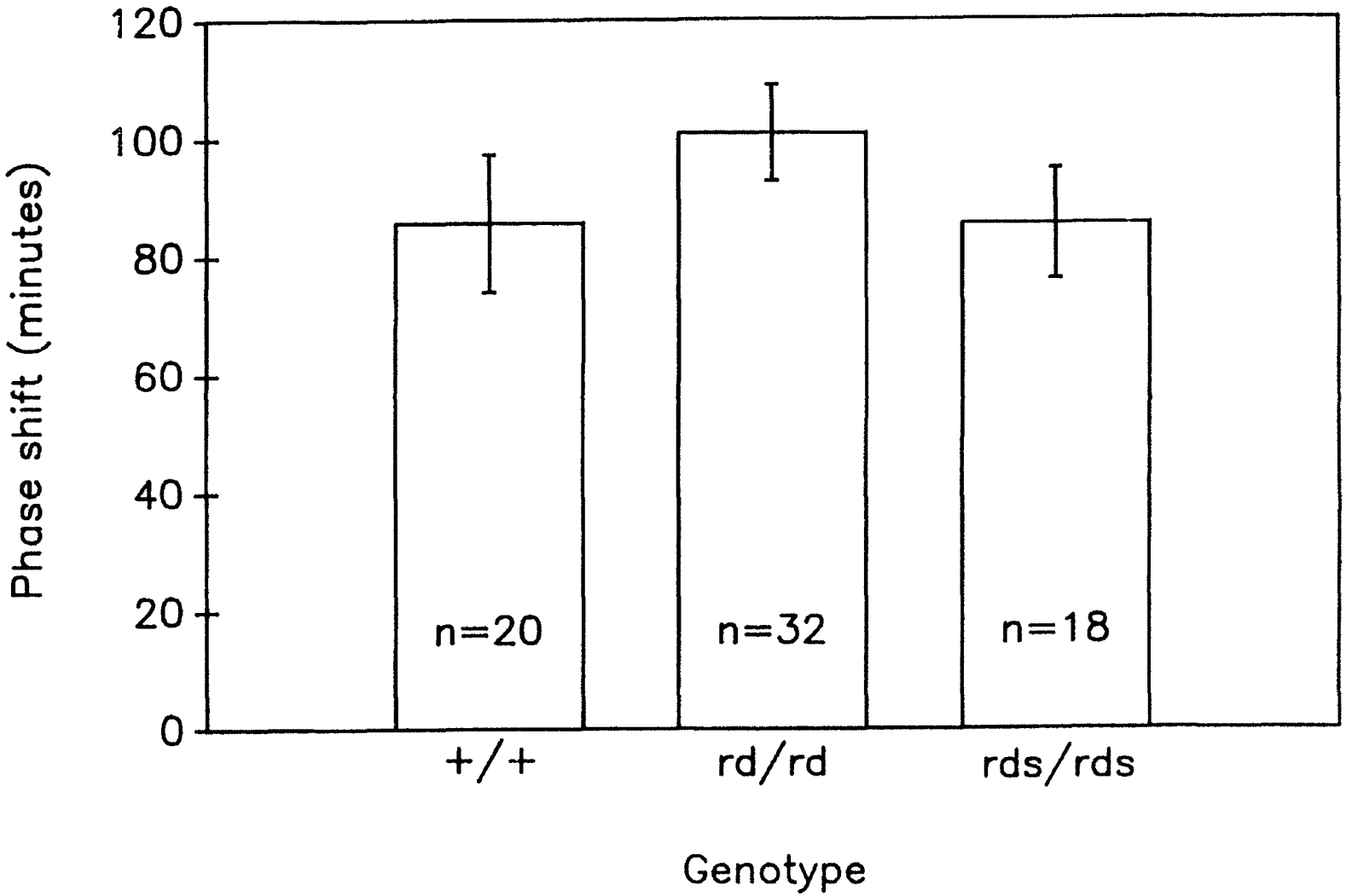
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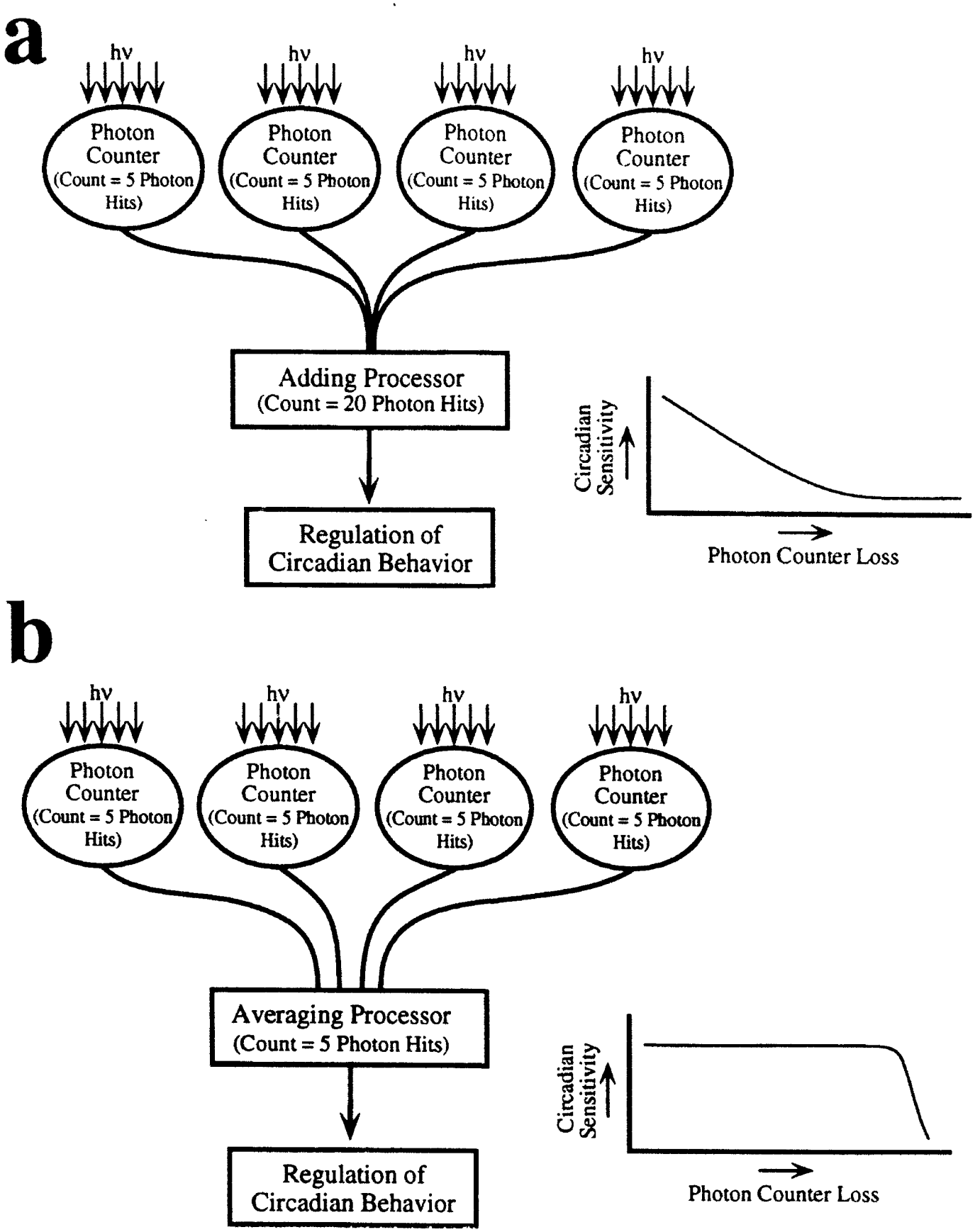
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